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End labeling of enzymatically decapped mRNA

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ABSTRACT

A method is presented for rapid and efficient 5' end labeling with ^{32}P of capped mRNAs, by a series of three enzymatic reactions: the blocking nucleotide of the cap structure is removed by tobacco acid pyrophosphatase, and after dephosphorylation with alkaline phosphatase the 5' end is labeled with γ-^{32}-P-ATP and T4 polynucleotide kinase.

INTRODUCTION

As in the case of DNA, end-labeling of RNA at the 5' terminus with γ-^{32}P-ATP and T4 polynucleotide kinase is an extremely useful procedure. It generates highly labeled molecules that can be used as molecular markers and hybridization probes; because of its greater stability, ^{32}P-end-labeled RNA is preferable to radioiodinated RNA[2,3], except for experiments involving in situ hybridization or S1-nuclease assays. Perhaps most importantly, ^{32}P-end-labeled RNA can be sequenced directly[4,5].

End-labeling of most eukaryotic and viral mRNA molecules is not straightforward, because of the nucleotide that blocks the 5' end in a "cap structure"[6]. The blocking nucleotide can be chemically removed by periodate oxidation followed by a β-elimination reaction[4,6], but this treatment is time consuming and relatively inefficient, often results in degradation and requires large amounts of mRNA. Recently, it was shown that the "cap structure" can be enzymatically removed without degradation of the polynucleotide chain[7-9] by use of a pyrophosphatase (tobacco acid pyrophosphatase, TAP) purified from cultured tobacco cells[10]. In this paper we describe a rapid and simple

procedure for 5' labeling of mRNA, after enzymatic removal of the "cap structure" by TAP.

MATERIALS AND METHODS

Purification of Enzymes

a) TAP. Callus cultures from stem pith of tobacco plants (Nicotiana tabacum var. Wisconsin 38) were initiated on the B5-medium of Gamborg[11], but with iron-EDTA instead of sequestrene-iron[12], and supplemented with 10 g/L agar. The callus can be maintained at 25° by subculturing each month, or the cells can be adapted to suspension growth in the same medium without agar, by incubation in the dark at 25° on a rotatory shaker at 100 rpm. The suspension cultures are maintained by inoculating fresh medium with 0.05 volumes of 14-day-old cultures. Cells are harvested after 7 days of growth by filtration, and stored frozen at -80° until enzyme extraction. The yield is 60 g wet weight per liter of culture.

TAP is purified from frozen cells according to the procedure described by Shishi et al[10], slightly modified and abbreviated as follows. All operations are carried out in the cold room. Seven to 10 g (wet weight) of frozen cells are suspended into 10 ml 0.1 M Na acetate pH 5.0, 0.2 M NaCl, 10 mM 2-mercaptoethanol and 1 mM EDTA, and homogenized in the stainless steel chamber (50 ml capacity) of a Sorvall Omni-Mixer at maximum speed (16,000 rpm) for 3 min. The homogenate is centrifuged at 3,000xg for 20 min and the supernatant is centrifuged again at 30,000xg for 20 min. To the supernatant of the second centrifugation solid ammonium sulfate (52 g/100 ml supernatant) is added slowly with stirring, within 30 min. After 30 more min of stirring and centrifugation at 30,000xg for 15 min, the pellet is collected and dissolved into 1 ml of Buffer A (10 mM Tris-HCl pH 7.5, 10 mM 2-mercaptoethanol and 0.01% Triton X-100). It is then dialyzed overnight against 2 L of the same buffer. The solution is recovered from the dialysis bag and centrifuged at 30,000xg for 15 min. The supernatant is then passed over a 1 ml DEAE-cellulose column equilibrated and developed with Buffer A. The effluent is collected and passed over a 1 ml phosphocellulose column equilibrated with Buffer A. The column

is washed with 10 ml of Buffer A containing 0.04 M NaCl, and the enzymatic activity is eluted under air pressure with Buffer A containing 0.12 M NaCl and 50% glycerol. Three 1 ml fractions are collected and assayed as described in the Results; most of the enzymatic activity is found in the first two fractions. Typically, 1 µl of the first fraction degrades 1-2 nmole ATP in 30 min. The fractions are stored at -20°. Freezing and thawing destroys the activity (T. Sugimura, personal communication and our own observations). At this stage of purification the preparation is RNase-free, as shown by treatment of homogeneously labeled rRNA with the enzyme preparation, followed by polyacrylamide gel electrophoresis (data not shown).

b) Bacterial alkaline phosphatase (BAP). RNase (and DNase) activity contaminating BAPF (Worthington) can be eliminated as described by Wimmer[13], according to the following detailed protocol (brought to our attention by W. Jelinek). Fifty units enzyme (spun out from ammonium sulfate) are dissolved into 6 ml 10 mM triethanolamine-HCl, pH 7.4, 5 mM $ZnCl_2$, and stirred vigorously for 1 hr on ice, in the cold room, after addition of 3 ml diethylpyrocarbonate (DEP). After brief centrifugation the upper (aqueous) layer is removed and extracted six times with diethylether. The remaining ether is evaporated with N_2. Most of the enzymatic activity survives the DEP treatment. One µl of the BAP solution, after treatment, hydrolyzes 1 to 5 nmoles ATP in 30 min at 37° (1 to 5 ATPase units). In most of the experiments reported here, however, we used BAPF purified as described by Efstratiadis et al[14] which is also RNase-free. An alternative to BAP which we have found convenient is calf intestine phosphatase which has 10 to 20-fold higher specific activity than BAP and can be easily purified free of RNase as follows. A suspension of the enzyme in ammonium sulfate (0.4 mg, 160 units; Boehringer Mannheim) is briefly centrifuged. The pellet is dissolved in 0.1 ml H_2O and passed over a Sephadex G-75 column (0.7 x 30 cm) equilibrated and developed with 20 mM Tris-HCl, pH 8.4, 100 mM KCl. Fractions (0.15 ml) are collected and 1 µl aliquots are assayed as described[15] or as described below for TAP but in 10 mM Tris-HCl, pH 8.0. The peak fractions which elute in the void volume are pooled, mixed with an equal volume

of glycerol, and stored at -20°. The enzyme is free of RNase as shown by the assay described above for TAP.

c) T4 polynucleotide kinase, free of RNase, was prepared as described by Panet et al[16] with the modifications described below. The enzyme was assayed as described by Richardson[17]. The pooled fractions of the phosphocellulose column were dialyzed for a total of 8 hours against 2 changes (4 L each) of 10 mM K phosphate, pH 7.5 containing 10 mM 2-mercaptoethanol and 0.01 mM ATP. The dialysate was concentrated by loading on a second phosphocellulose column (1.2 x 3.0 cm) and eluting with 0.5 M KCl in the above buffer. The peak fractions were pooled (6.0 ml, 55,000 units), concentrated to a final volume of 3.0 ml with Ficoll, and loaded onto a Sephadex G-100 column (3.0 x 35.0 cm) equilibrated and eluted with 50 mM Tris-HCl, pH 7.6 containing 100 mM KCl, 5 mM DTT, 5% sucrose (RNase free, Schwartz Mann) and 0.01 mM ATP. The peak fractions (24 ml containing 48,000 units) were pooled and concentrated to approximately 1/3 the volume with Ficoll. The enzyme was then dialyzed against 50 mM Tris-HCl, pH 7.6 containing 100 mM KCl, 2 mM DTT, 0.01 mM ATP and 50% glycerol, and stored at -20° where it is stable for years. The final concentration was 4 units/µl with a yield of 25,000 units.

Other methods

Rabbit globin mRNA was purified as described[18]. We consistently observed a differential loss of α-globin mRNA by using this method. γ-^{32}P-ATP was prepared at a specific activity of about 1000 Ci/mmole as described[19]. Conditions of polyacrylamide gel electrophoresis are described in figure legends.

RESULTS AND DISCUSSION

Enzyme Assay

In preliminary experiments it was observed that TAP has comparable specific activities against ATP and against the cap structure of ^{3}H-methyl-labeled VSV RNA (data not shown). Thus, the enzyme can be conveniently assayed by its ATPase activity. We recommend the following simple procedure for assay of the enzyme and determination of its titer (Fig. 1.

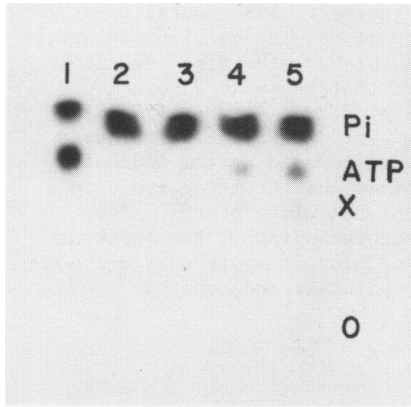

Figure 1. Assay of TAP by its ATPase activity, and determination of the titer. Reactions were 10 µl each (see Text) and contained 1 µl of enzyme, except for the blank which contained none (Slot 1). The ATP present in the reaction was a constant amount of γ-^{32}P-ATP (3 pmoles, 1000 Ci/mmole; contaminated with ^{32}P-phosphate), plus unlabeled ATP to a final amount of 1 nmole (Slot 2), 1.5 nmole (Slot 3), 3 nmole (Slot 4), 5 nmole (Slots 1 and 5). After 30 min at 37°, 1 µl of the reaction mixtures were spotted and chromatographed on a PEI-cellulose plate. The origin, ATP and inorganic phosphate are indicated by O, ATP and Pi, respectively. X indicates a transient product, presumably inorganic pyrophosphate.

A stock ATP solution is made by adding 0.5 µl γ-^{32}P-ATP (5 pmoles, 100 Ci/mmole) to 100 µl H_2O. A 10 µl assay mixture contains 8 µl ATP solution, 1 µl 10X TAP buffer (0.5 Na acetate, pH 6.0, 0.1 M 2-mercaptoethanol) and 1 µl enzyme. It is incubated for 10 min or more at 37° and then 1 µl aliquots are spotted on a PEI-cellulose plate, which is developed in 0.75 M K phosphate adjusted to pH 3.5 with phosphoric acid. Larger aliquots cannot be spotted because of artifacts caused by glycerol. The disappearance of the ATP spot is followed by autoradiography. For quantitation, the substrate and product spots are cut out separately and counted by liquid scintillation. The titer of the enzyme is found by diluting the ATP solution with increasing amounts of unlabeled ATP, and is expressed in ATPase units (1 unit hydrolyzes 1 nmole ATP in 30 min).

Time Course

Routinely, we use a volume of enzyme solution which, according to its titer, is more than enough to bring about complete hydrolysis of the substrate in 10 to 30 min. If necessary, lower amounts of enzyme can be used provided the reaction time is extended. Figure 2 shows that an amount of enzyme 5 times lower than that necessary for completion of the reaction in 30 min is able to hydrolyze 96% of the substrate by 2 hr. The kinetics of the reaction appear to be first order. However,

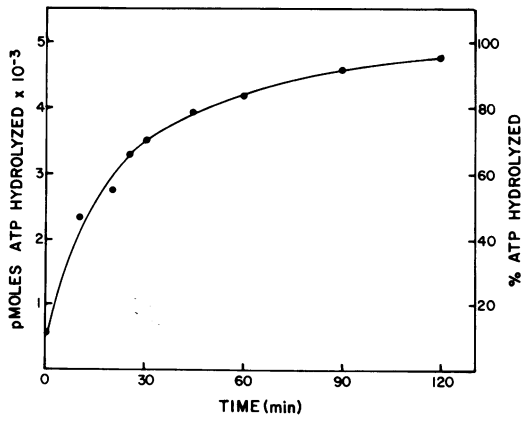

Figure 2. Time course of a TAP reaction. A reaction mixture, 20 µl but otherwise identical to that shown in Figure 1, Slot 5, was incubated for 120 min at 37°, and 1 µl aliquots were removed at the indicated times and chromatographed on a PEI-cellulose plate. After autoradiography, the substrate and product spots were cut out separately and counted.

reactions with very low amounts of enzyme should be avoided, since they tend not to reach completion.

End-labeling of mRNA

End-labeling is performed in three steps. The 7-methyl guanine of the cap structure is first removed by TAP[7-9]. The exposed 5' end is then dephosphorylated using alkaline phosphatase (bacterial or from calf intestine). Finally, a ^{32}P-phosphate is added to the 5' end using γ-^{32}P-ATP as a donor and T4 polynucleotide kinase.

For the TAP reaction 1 µl enzyme, 1 µl 10X TAP buffer, RNA (usually 5 to 50 pmoles 5' end) and H_2O to 10 µl are mixed and incubated for 30 min at 37°. To this mixture are then added 2 µl of 0.5 M Tris-HCl, pH 8.3 (at 37°), BAP in excess for removal of the calculated amount of 5' phosphates, and H_2O to a final reaction volume of 20 µl. The mixture is incubated for 30 min at 37°. One µl of 250 mM K phosphate, pH 9.5 is then added; it inhibits TAP[10] and competitively inhibits BAP[20,21]. We have observed that 10 mM K phosphate will inhibit completely BAP (5 ATPase units) even in the presence of moderately high substrate concentrations (0.2 mM ATP). Although phosphate also partially inhibits the kinase[22], this inhibition can be overcome by using excess enzyme. Because of convenience and avoidance of losses, this procedure of phosphatase inactivation is much preferable to phenol extraction. After addition of the phosphate, the mix-

ture is stirred thoroughly and transferred to a tube containing dry γ-^{32}P-ATP (200 to 500 pmoles, 1000 Ci/mmole, dried under a stream of N_2). To this are added 1 µl 250 mM $MgCl_2$, 2 µl 50 mM DTT and 1 µl (4 units) kinase. Incubation is for 30 to 60 min at 37°.

For precipitation of the labeled RNA without precipitation of the enzymes, we add 100 µl of 2 M ammonium acetate, 50 µg tRNA as carrier and 300 µl ethanol[19]. The mixture is chilled for 10 min in a Dry-Ice acetone bath and spun at 12,000xg for 10 min. For electrophoresis, the pellet of labeled RNA is dissolved in 10 µl of 20 mM EDTA, 40 ml deionized formamide is added, and the sample is heated to 75° for 1 min before being layered on the gel.

Fig. 3 shows rabbit globin mRNA end-labeled by this procedure and analyzed by electrophoresis on a 6% polyacrylamide, 7 M urea gel. The major mRNA species is intensely labeled and its size corresponds to that of β-globin mRNA. The mRNA preparation used was known to be highly enriched in β-globin mRNA, as shown by cell-free translation[23].

mRNA Sequencing

The labeled mRNA was eluted[24] from the gel shown in Fig. 3. The positions of adenine, guanine, and the pyrimidines with respect to the labeled 5' terminus were obtained using partial base-specific nuclease and alkaline digestions[5]. The reaction products were electrophoretically fractionated by size under denaturing conditions in a polyacrylamide gel and the sequence directly determined by autoradiography (Fig. 4). The derived sequence (approximately 30 nucleotides) is in complete agreement with the known sequence of the 5' terminus of rabbit β-globin mRNA[4,14]. From the sequence displayed in Fig. 4 it is apparent that a unique 5' terminus was labeled. If significant 5'-exonuclease digestion had occurred during any of the enzymatic reactions prior to sequencing, a mixture of labeled molecules would have been recovered from the preparative gel (Fig. 3), since fragments differing in length by only a few nucleotides would not have been resolved. The products of the sequencing reactions would have reflected such a mixture, i.e. some bands

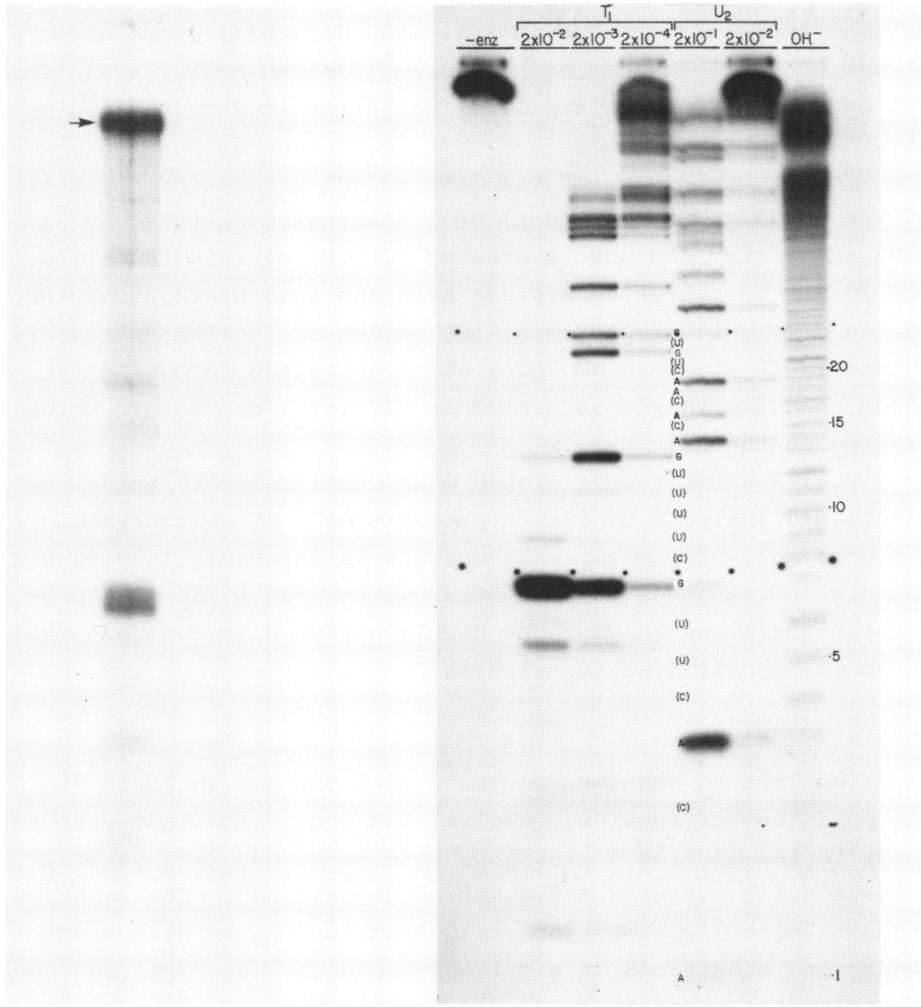

Figure 3 (left). Autoradiogram of ^{32}P-end-labeled globin mRNA electrophoresed on a 6% polyacrylamide gel (1:30 bis-acrylamide) containing 50 mM Tris-borate, pH 8.3, 1 mM EDTA and 7 M urea. The material of the band indicated by an arrow was eluted from the gel and sequenced.

Figure 4 (right). Autoradiogram of the 5' terminal sequence of rabbit β-globin mRNA, end-labeled with ^{32}P and recovered from the gel shown in Fig. 3. Partial nuclease digestion was obtained by ten-fold serial dilution of RNase T_1 (G-specific) or U_2 (A-specific) into 20 µl aliquots of a buffer containing 20 mM Na citrate pH 5.0, 1 mM EDTA, 7 M urea, 0.25 mg/ml tRNA, 0.025% xylene cyanol, bromophenol blue and labeled mRNA incubated at 50° for 15 min. For limited alkaline hydrolysis labeled mRNA was incubated in 20 µl 50 mM $NaHCO_3$/ Na_2CO_3 pH 9.0, 1 mM EDTA, 0.25 mg/ml tRNA in a sealed capillary at 90° for

would have been present at the same position in both the (A) and (G)-specific lanes of the gel.

In conclusion, TAP is easy to prepare at a purity and titer high enough for convenient and specific removal of the nucleotide blocking the 5' end in the cap structure of eukaryotic mRNAs. The exposed 5' end can then be labeled by kinase to a high specific activity (up to 1 μCi/pmole end). The labeled RNA is free of degradation products (Figs. 3 and 4). It can be sequenced directly and, of course, it can be used as a labeled marker and as a probe in hybridization reactions. We were unable to obtain undegraded end-labeled rabbit globin mRNA using T4 polynucleotide kinase itself for removal of the cap structure[25,26].

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15 min and then 20 μl 10 M urea, 0.05% xylene cyanol and bromophenol blue were added. The reaction products were layered in adjacent lanes on a 20% polyacrylamide, 7 M urea gel, and electrophoresed for 4 hr at 1000 volts. The gel was exposed to x-ray film for 12 hr at -20°. Numbers above the slots indicate units enzyme per 5 μg RNA. The numbers along the last lane (products of limited alkaline hydrolysis) denote fragment length (in nucleotides).

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